植物研究雑誌
J. Jpn. Bot.
81: 139–147 (2006)

Morphology and Molecular Phylogeny of *Gonium multicoccum* (Volvocales, Chlorophyceae) Newly Found in Japan

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(Received on December 5, 2005)

Strains of *Gonium* were isolated from a soil sample collected in Fukuoka Prefecture, Japan. Morphological observations identified the strains as *Gonium multicoccum* Pocock, which has not been reported previously from Japan. Molecular phylogenetic analyses based on the plastid *rbc*L gene sequences suggested a close relationship between Japanese and Nepalese strains of the species.

Key words: Chlorophyceae, *Gonium multicoccum*, ITS2, *rbc*L, Volvocales.

The genus *Gonium* O. F. Müll. (Goniaceae, Volvocales, Chlorophyceae) is characterized by a flattened colony with 8, 16, or 32 biflagellate cells arranged in a single layer (Nozaki and Ito 1994, Nozaki 2003). There are seven species in the genus (Watanabe 1977, Ettl 1983, Nozaki 1989). There are detailed morphological reports on the strains of four species grown in culture [*G. pectorale* O. F. Müll. (Stein 1958, Kusumoto et al. 1978), *G. multicoccum* Pocock (Nozaki and Kuroiwa 1991), *G. quadratum* E. G. Pringsh. ex Nozaki (Nozaki 1993), and *G. viridistellatum* M. Watanabe (Watanabe 1977, Nozaki 1989)]. The following species have been recorded in Japan: *G. pectorale* (Kusumoto et al. 1978, Nozaki 1984), *G. viridistellatum* (Watanabe 1977, Nozaki 1989), and *G. formosum* Pascher (Akiyama et al. 1977).

Recently, strains of *G. multicoccum* were isolated from soils collected in Fukuoka Prefecture, Japan. Here, we report the morphology, reproduction, and molecular phylogeny of these strains.

Materials and Methods

The soil samples used in this study were collected in a paddy field at Chikuzen-machi, Asakura-gun, Fukuoka Prefecture, Japan in March 2004. Clonal cultures (Asa.Goni.84, Asa.Goni.6, and AsCl-1) were established using the pipette-washing method (Pringsheim 1946) from Petri dishes (90 × 20 mm) in which a small amount of dried soil sample had been rewetted with distilled water. The cultures were grown in screw-cap tubes (18 × 150 mm) containing about 11 mL of AF-6 medium (Kato 1982) modified by the elimination of $CaCO_3$ and the addition of 400 mg·L^{-1} MES (Kasai et al. 2004). The cultivation temperature was about 25°C, with alternating periods of 10 h darkness and 14 h light at an intensity of

150–200 μmol·m^{-2}·s^{-1} provided by cool-white fluorescent lamps.

To observe the vegetative morphology and asexual reproduction, about 0.5 mL of an actively growing culture was inoculated into fresh medium every seven to eight days. To induce sexual reproduction, 22 mL of four-day-old cultured material were condensed to 0.5–1.0 mL by centrifugation. Subsequently, 2.0 mL of AFM medium (Nakazawa et al. 2001) were added to the condensed culture in a watch glass (60 mm in diameter) supported on a glass depression in Petri dishes. To minimize evaporation from the watch glasses, about 5.0 mL of distilled water were added to the bottoms of the Petri dishes. The Petri dishes were exposed to the growing conditions described above. Light micros-

Table 1. List of *rbc*L gene and ITS2 sequences used in this study

Taxa	Strain designation	Origin and DDBJ/EMBL/GenBank accession number	
		*rbc*L	ITS2
Goniaceae			
Gonium multicoccum	UTEX* 2580	Nozaki et al. (1995) D63435	Mai and Coleman (1997) U66966
	UTEX 783	Nozaki et al. (2002) AB076102 & AB076103	Mai and Coleman (1997) U66967
	NIES**-1038	This study AB246187	This study AB246191
	Asa.Goni.84 (NIES-1708)	This study AB246188	This study AB246192
Gonium octonarium	NIES-851	Nozaki et al. (1995) D63436	
Gonium pectorale	NIES-569	Nozaki et al. (1995) D63437	
	Kita.Goni.3*** (NIES-1713)	This study AB246189	
	Kaneko4**** (NIES-1711)	This study AB246190	
Gonium quadratum	NIES-653	Nozaki et al. (1995) D63438	
Gonium viridistellatum	NIES-289	Nozaki et al. (2002) AB076091	
	NIES-654	Nozaki et al. (1995) D86831	
	NIES-857	Nozaki et al. (2002) AB076092 & AB076093	
Volvocaceae			
Pandorina morum	UTEX 880	Nozaki et al. (2000) AB044166	
Pandorina colemaniae	NIES-572	Nozaki et al. (1995) D63441	

*Culture Collection of Algae at the University of Texas at Austin (Starr and Zeikus 1993).
**Microbial Culture Collection at the National Institute for Envioromental Studies (Kasai et al. 2004).
***Isolated from soil collected in a paddy field, Kitahiroshima, Hokkaido, Japan, in March 2004.
****Isolated from soil collected in a paddy field, Kin-cho, Okinawa, Japan, in March 2000.

copy was carried out using an Olympus BX60 microscope equipped with Nomarski interference optics.

The methods for extracting total DNA and sequencing the large subunit of the Rubisco (*rbc*L) gene (including interrupted group I introns) of two strains of *G. multicoccum* (Asa.Goni.84 and NIES-1038) and two new isolates of *G. pectorale* (Kita.Goni.3 and Kaneko4) (Table 1) were essentially as described previously (Nozaki et al. 1995, 1998, 2000, 2002). The region sequenced corresponded to positions 31–1158 of the *Chlorella vulgaris* Beij. *rbc*L gene (Yoshinaga et al. 1988, Wakasugi et al. 1997). The new Japanese strain of *G. multicoccum* described here had two interrupted group I introns in the same exon positions as the Nepalese strain (UTEX 2580) of *G. multicoccum* (Nozaki et al. 2002). The coding regions (1128 bp) of the sequences from these four strains were aligned with those of eight other *Gonium* strains and two strains of the related genus *Pandorina* (Table 1). From this alignment, a distance matrix was calculated by applying the two-parameter method (Kimura 1980) in Clustal X (Thompson et al. 1997). A phylogenetic tree was constructed using the neighbor-joining (NJ) algorithm (Saito and Nei 1987), also using Clustal X, and the robustness of the resulting lineages was tested using a bootstrap analysis (Felsenstein 1985) with 1,000 replications. In addition, a maximum parsimony (MP) analysis [including a bootstrap analysis based on 1,000 replications of the general heuristic search using the tree-bisection-reconnection (TBR) branch-swapping algorithm] was performed using PAUP* 4.0b10 (Swofford 2003). Since the genus *Gonium* has been resolved as a monophyletic group (Nozaki et al. 2000, 2002), the two *Pandorina* strains (Table 1) were designated as the outgroup. In addition, the internal transcribed spacer (ITS) 2 regions of ribosomal DNA from two strains of *G. multicoccum* (Asa.Goni.84 and NIES-1038) were sequenced, as described by Coleman et al. (1994).

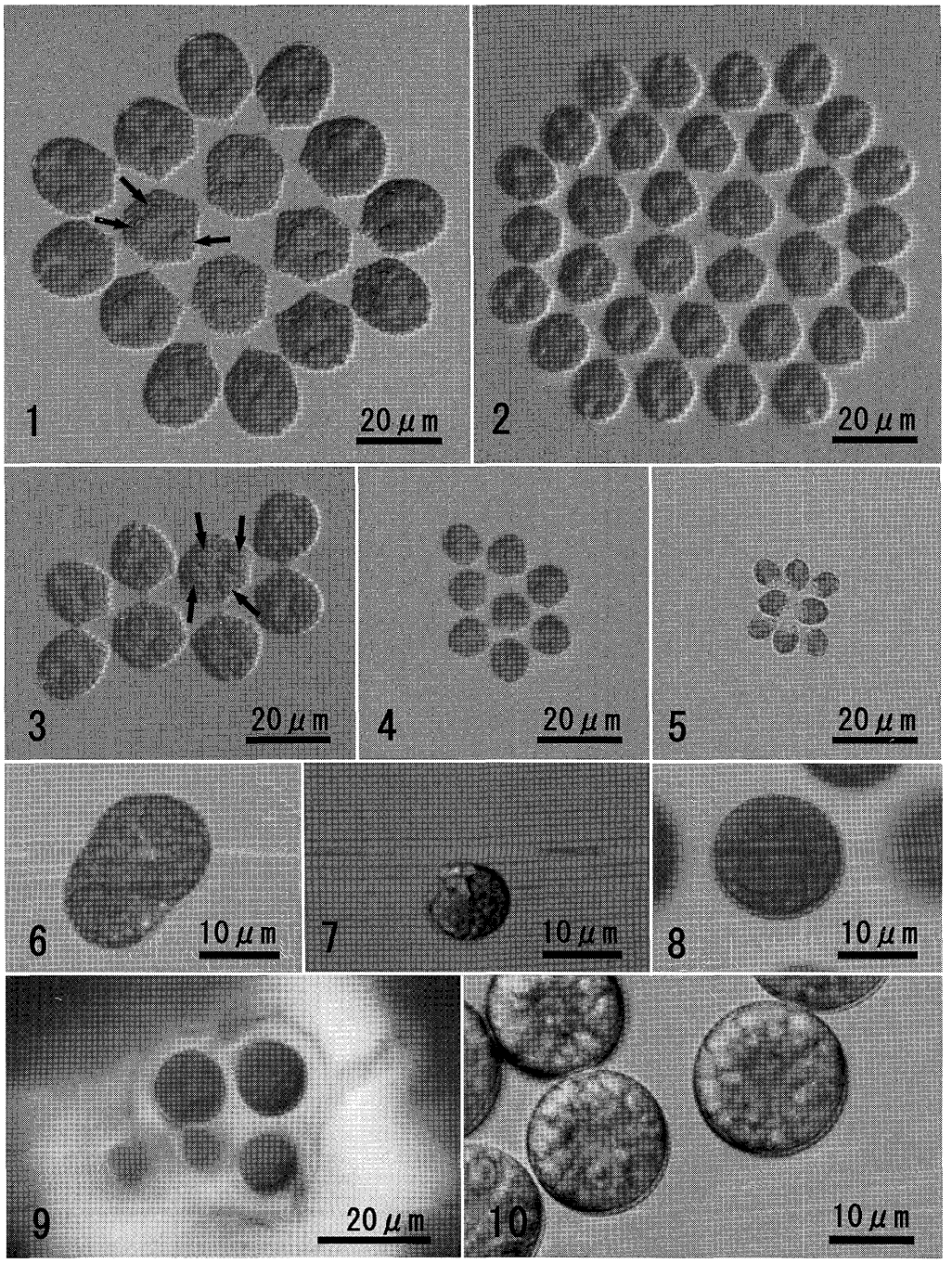
Results

The vegetative colonies were composed of 8, 16, or 32 cells. In four- to eight-day-old cultures, 16-celled colonies were most abundant. In culture more than 10 days old, 32-celled colonies were not formed, but 8-celled colonies became frequent. The 16-celled colonies contained 12 peripheral and four central cells arranged in a curved, rhomboid to square plate, measuring up to 99 μm wide (Fig. 1). The 32-celled colonies were octagonal in outline, radially symmetrical, and contained 4 central, 10 median, and 18 peripheral cells in a plate measuring up to 135 μm wide (Figs. 2, 11). The cell arrangement of the 8-celled colonies may be classified into two types. Approximately 95 % of the 8-celled colonies had cells arranged in four zigzag rows of two cells each (Fig. 3). In the other 8-celled colonies, the cells were arranged in two rows of three cells each, with a marginal (Fig. 4) or central (Fig. 5) row of two cells.

The cells were nearly spherical, elongate bell-shaped, or angular in shape, and were up to 21 μm long. Each cell was biflagellate and had a stigma in the anterior portion, two contractile vacuoles near the base of the flagella, and a massive cup-shaped chloroplast. In the young colonies, each cell contained one to three pyrenoids, whereas in the mature colonies, two to eight pyrenoids were seen in the chloroplast (Figs. 1–5).

Asexual reproduction was accomplished by autocolony formation. Each cell of the 8-, 16-, or 32-celled colonies divided three, four, or five times successively to form an 8-, 16-, or 32-celled daughter colony, respectively, within the parental individual gelatinous sheath.

The strains were homothallic and exhibited isogamous sexual reproduction. The



Figs. 1–10. Nomarski interference microscopy of Japanese strain of *Gonium multicoccum* Pocock (Asa.Goni.84). Fig. 1. 16-celled vegetative colony. Note multiple pyrenoids (arrows). Fig. 2. 32-celled vegetative colony. Figs. 3–5. Three types of 8-celled vegetative colonies. Note multiple pyrenoids (arrows). Figs. 6–10. Sexual reproduction. Fig. 6. Late stage of plasmogamy. Fig. 7. Quadriflagellate zygote. Fig. 8. One-day-old aplanozygote. Fig. 9. Six-day-old aplanozygotes surrounded by watery gelatinous matrix. Indian ink preparation. Fig. 10. Eight-day-old mature aplanozygotes.

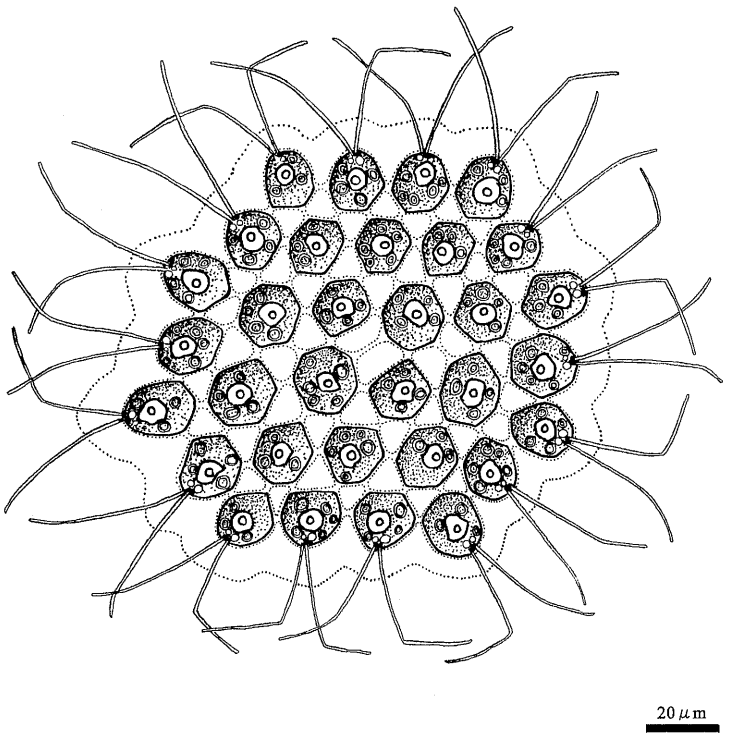


Fig. 11. Line drawing of vegetative colony of Japanese strain of *Gonium multicoccum* Pocock.

mating reaction occurred within 2 days after transferring the colonies to AFM medium. The colonies dissociated into individual cells, from which biflagellate protoplasts escaped to become gametes. After gametic union (Fig. 6), a quadriflagellate planozygote was formed (Fig. 7). The zygotes then settled and secreted a cell wall (Fig. 8). A broad gelatinous matrix could be seen around them (Fig. 9). After about one week, the hypnozygotes became reddish-brown in color. They measured 14–26 μm in diameter (Fig. 10).

Our Japanese strain (Asa.Goni.84) and a strain of *G. multicoccum* from Nepal (UTEX 2580; Nozaki et al. 2002) had identical sequences of the *rbc*L exon (1128 bp) and group I introns (844 bp). However, the ITS2 sequences (224–226 bp) differed (Fig. 12).

In the phylogenetic analyses of the *rbc*L gene sequences, the Japanese strain and three other strains of *G. multicoccum* formed a monophyletic group (with 93 % and 86 % bootstrap values in NJ and MP analyses, respectively) that is separated from four other species of *Gonium*. *G. multicoccum* was subdivided into two clades (Fig. 13). One comprised two North American strains (NIES-1038 from Texas and UTEX 783 from California), while the other contained the Japanese and Nepalese strains (UTEX 2580). Both clades were supported with 100 % bootstrap values in the NJ and MP methods (Fig. 13).

Discussion

Pocock (1955) characterized *Gonium multicoccum* as having 8-, 16-, or 32-celled

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                                20                  40
Asa.Goni.84 : AATACTCGCTCCCTATATACATG--------GGGAACGGAACTGACTGTC
UTEX2580    : AATACTCGCTCCCTATATACATG--------GGGAACGGAACTGGCTGTC
UTEX783     : AATACTCGCTCCCTCCAACTCCGGTTGGTTAGGGAACGGAACTGGCTGTC
NIES-1038   : AATACTCGCTCCCTCCAACTCCGGTTGGTTAGGGAACGGAACTGGCTGTC

                    60                  80                  100
Asa.Goni.84 : TCGGCGGTTAATTTAA---TTAACTGCCGGGTCAGTTGAAGTATGCAGAG
UTEX2580    : TCGGCGGTTA-TTTAA---TTAACTGCCGGGTCAGTTGAAGTATGCAGAG
UTEX783     : TCGGCAGTTAA-CTAACCGTTAACTGCCGGGTCAGCTGAAGTGTGCAGAG
NIES-1038   : TCGGCAGTTAA-CTAACCGTTAACTGCCGGGTCAGCTGAAGTGTGCAGAG

                           120                 140
Asa.Goni.84 : GTTATTGCATGGACCCGTTTATGGGCCTCTACTGGGTAGGCAACTTGC-T
UTEX2580    : GTTATTGCATGGACCCGTTTATGGGCCTCTACTGGGTAGGCAACTTGC-T
UTEX783     : GTTGATGCATGGACCCGCTCATGGGCCTCTACTGGGTAGGCAATTAACTT
NIES-1038   : GTTGATGCATGGACCCGCTCATGGGCCTCTACTGGGTAGGCAATTAACTT

                    160                 180                 200
Asa.Goni.84 : TGCTAATGCTTTAGTAGATGGCCTGGGACCGTGTGTGTTTTAACCCAAAC
UTEX2580    : TGCTAATGCTTTAGTAGATGGCCTGGGACCGTGTGTGTTTTAACCCAAAC
UTEX783     : TGCTAATGCTTTAGTAGATGGCTTGGGACTGTGCCTGTC--GGCCCAAAC
NIES-1038   : TGCTAATGCTTTAGTAGATGGCTTGGGACTGTGCCTGTC--GGCCCAAAC

                           220                 240
Asa.Goni.84 : CAGGAACTCAAT-ATTTTATTGAGCAACTGT--TTATTTTC
UTEX2580    : CAGGAACTCAAT--TTTCATTGAGCAACTGT--TTATTTTC
UTEX783     : CAGGAACTCAGTTTACGGACTGAGCAACTGTACCTATTT-C
NIES-1038   : CAGGAACTCAGTTTACGGACTGAGCAACTGTACCTATTTTC
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Fig. 12. Alignment of ITS2 sequences from four strains of *Gonium multicoccum* Pocock (Table 1). Nucleotides which are different between Asa.Goni.84 and UTEX 2580 are indicated by shading.

vegetative colonies with multipyrenoid cells. Although *G. discoideum* Prescott (1942) has 32-celled colonies, the number of pyrenoids in the chloroplast and the shape of vegetative cells in this species differ from those of *G. multicoccum*. In *G. discoideum*, there are usually two pyrenoids per cell, and the cells are pyriform (Prescott 1942, Ettl 1983). By contrast, *G. multicoccum* has two to 12 pyrenoids in mature vegetative cells, which are almost spherical or elongate (Pocock 1955, Nozaki and Kuroiwa 1991). Accordingly, our Japanese collection can be assigned to *G. multicoccum*.

Pocock (1955) reported the frequent production of 8-celled colonies, in which the cells were arranged in two rows of three, and a row of two. By contrast, our Japanese alga rarely produced 8-celled colonies, and these had two cell arrangements, one of which was characteristic of *G. multicoccum*, as described by Pocock (1955) (Figs. 4, 5), while the other resembled the arrangement of *G. pectorale* (Fig. 3; Nozaki 1984, 1988). Nozaki and Kuroiwa (1991) reported both of these 8-celled colony arrangements in Nepalese strains of *G. multicoccum*, and our *rbc*L gene tree demonstrated a close relation-

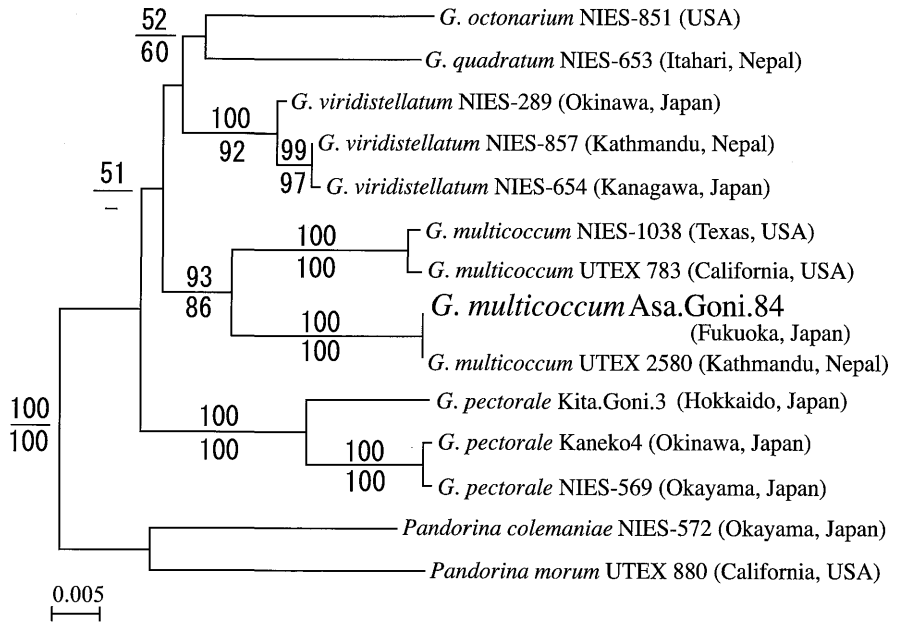

Fig. 13. Neighbor-joining (NJ) tree based on 1128 bp of the *rbc*L genes from 12 strains of five *Gonium* (*G.*) species and two strains of *Pandorina* (Table 1). Branch lengths are propotional to Kimura (1980) distances, which are indicated by the scale bar below the tree. Numbers above or below the branches represent 50 % or more bootstrap values based on 1000 replications of the NJ or maximum parsimony analyses, respectively.

ship between our Japanese strain of *G. multicoccum* and the Nepalese strain (UTEX 2580) that Nozaki and Kuroiwa (1991) studied (Fig. 13). Furthermore, the sequence of the *rbc*L gene [including two interrupted group I introns (1972 bp)] of our Japanese strain exactly matched that of the Nepalese strain (UTEX 2580; Nozaki et al. 2002). This indicates a very recent radiation of these two strains of *G. multicoccum*, and there is no genetic differentiation between them. However, there were differences in the ITS2 sequences between the two strains (Fig. 12).

We are grateful to Dr. M. Kato of National Science Museum, and Dr. Y. Kita of University of Tokyo for their helpful suggestions. We want to thank all the members of Laboratory of Plant Systematics of Department of Biological Science, Graduate School of Botany, University of Tokyo for their kind supports. We are also grateful to Ms. S. Tsuda of University of Tokyo for her providing soil materials. This study was supported in part by a grant from the Institute for Fermentation, Osaka (to H. N.).

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山田敏寛[a*]，仲田崇志[b]，宮地和幸[a]，野崎久義[b]：

日本新産 *Gonium multicoccum*（緑藻綱，オオヒゲマワリ目）の形態と分子系統

福岡県朝倉郡筑前町の水田土壌より *Gonium* を分離・培養し，光学顕微鏡による形態観察を行った結果，これまでに日本より報告のない *Gonium multicoccum* と同定された．葉緑体 *rbc*L 遺伝子を用いた分子系統解析を実施した結果，本藻はネパール産の本種と最も近縁であることが判明した．

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